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Stool immune profiles evince gastrointestinal inflammation in Parkinson's disease

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Abstract

Background—Gastrointestinal symptoms are common in Parkinson's disease and frequently precede the development of motor impairments. Intestinal inflammation has been proposed as a driver of disease pathology, and evaluation of inflammatory mediators in stool could possibly

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identify valuable early-stage biomarkers. We measured immune- and angiogenesis-related proteins in human stool to examine inflammatory profiles associated with Parkinson's disease.

Methods—Stool samples and subjects' self-reported metadata were obtained from 156 individuals with Parkinson's disease and 110 without, including spouse and non-household controls. Metadata were probed for disease-associated differences, and levels of 37 immune and angiogenesis factors in stool homogenates were measured by multiplexed immunoassay and compared across experimental groups.

Results—Parkinson's disease patients reported greater incidence of intestinal disease and digestive problems than controls. Direct comparison of levels of stool analytes in patients and controls revealed elevated Flt1, IL-1α, and CXCL8 in patients' stool. Paired comparison of patients and spouses suggested higher levels of multiple factors in patients, but this was complicated by sex differences. Sex, body mass index, a history of smoking, and use of probiotics were found to strongly influence levels of stool analytes. Multivariate analysis accounting for these and other potential confounders confirmed elevated levels of IL-1α and CXCL8 and additionally revealed increased IL-1β and CRP in stool in Parkinson's disease. These differences were not dependent on subject age or disease duration.

Conclusions—Levels of stool immune factors indicate that intestinal inflammation is present in patients with Parkinson's disease.

Keywords

inflammation; Parkinson's disease; intestine; stool; biomarker

Introduction

Parkinson's disease (PD) is currently defined by motor impairments such as resting tremor, bradykinesia, rigidity, and gait disturbance, but non-motor features including cognitive impairment, hyposmia, anxiety, depression, sleep disturbances, and, prominently, gastrointestinal (GI) dysfunction have been gaining increasing attention and have a profound impact on quality of life. Constipation is reported by approximately 50% of PD patients[1] and can be detected by objective measures in nearly 80%[2]. It frequently precedes the onset of motor symptoms by more than 15 years[3]. Pathological abnormalities including the PD-related aggregation of alpha synuclein (αSYN) have been identified in intestinal biopsies from PD patients[4] and even in subjects in the pre-motor phase of disease,[5, 6] although consensus on the best interpretation of these results has not been reached (reviewed in[7]). PD patients also exhibit significant differences in gut bacterial populations[8–16] and increased intestinal permeability compared to individuals without PD[17, 18]. With accumulating evidence that GI symptoms are present from the earliest stages of PD, it has been proposed that PD pathology may originate in the gut and later spread to the central nervous system (CNS). Immune activity may advance this progression, as intestinal inflammation can promote systemic and also CNS inflammation[19, 20] which contributes to PD-related neurodegeneration[21].

Intestinal symptoms observed in PD are consistent with conditions of inflammation. Constipation in PD patients is attributed to slow intestinal transit time[22, 23], a symptom

which is also frequently observed in other conditions involving chronic GI inflammation such as inflammatory bowel disease (IBD)[24] and obesity[25]. Mechanistically, this condition has been linked to intestinal immune activation[26] and to intestinal dysbiosis, which is another hallmark of disorders involving gut inflammation[24, 27]. Immune activation promotes increased αSYN expression and aggregation[17, 28, 29], and αSYN in turn stimulates proinflammatory immune responses[28, 30]. In keeping with the well-known connections between inflammatory stimuli and impairment of gut barrier function[17, 31], intestinal permeability in PD correlates with levels of αSYN as well as indicators of oxidative stress[18]. Additionally, recent studies have reported significant coincidence of IBD or irritable bowel syndrome and PD[32–34]. One study reported the most direct confirmation of intestinal inflammation in PD, finding increases in mRNA transcripts encoding four proinflammatory cytokines (tumor necrosis factor, interferon gamma, interleukin-6, and interleukin-1β) as well as three glial markers in colonic biopsies of PD patients compared to age-matched healthy controls[35].

Given the accumulation of evidence suggesting that pronounced GI dysfunction and dysbiosis occurs in PD, we conducted an extensive analysis of immune and angiogenesis factors in stool to assess the GI inflammatory state in PD patients, their healthy spouses, and unrelated healthy control subjects. We also evaluated the prevalence of certain PD-associated non-motor symptoms in our subject cohort and assessed how these and other physiological and lifestyle factors influenced levels of stool analytes.

Methods

Subjects

A subset of the NeuroGenetics Research Consortium subjects from Atlanta, GA; Albany, NY; and Seattle, WA were invited to participate (re-contacted based on prior Institutional Review Board-approved consents). These included most of the subjects evaluated for stool microbiota composition described by Hill-Burns *et al*[14], excluding those for whom stool samples were of insufficient quantity for this analysis. Of 266 subjects, 156 had been diagnosed with PD by a movement disorder specialist (PD patients), and 110 reported no diagnosis of PD (controls). Within the control group, 49 subjects were spouses of PD patients (household controls), 39 of whom had a spouse participating in this study, and 61 were not known to live with anyone diagnosed with PD (non-household controls). We evaluated whether any significant differences existed between household and non-household control groups in levels of stool analytes (Supp Table 1) or in various relevant metadata factors (Supp Table 2), and, finding none, we combined these groups for all analyses except a paired comparison of PD patients and their respective spouses. Over 99% of subjects described their race as “white.” Ages of participants ranged from 36 to 94 years; mean age of control subjects was 70.8 years (standard deviation 8.8 years), and mean age of PD subjects was 68.3 years (standard deviation 8.8 years). A strong male sex bias was observed in PD subjects, with 112 males and 44 females. Accordingly, the opposite sex bias was observed in household controls, with 36 females and 13 males. Non-household controls consisted of 35 males and 26 females. In addition to providing a stool sample, subjects

completed a questionnaire on demographics, health problems, medications, and dietary practices.

Processing of stool

Stool samples were collected on BBL CultureSwabs (Becton, Dickson and Company; Sparks, MD) by participants at home, shipped immediately via the United States Postal Service at ambient temperature, and then stored at −20°C until processing. Swab tips were placed in tubes with 5-mm stainless steel beads (QIAGEN, Valencia, CA) and 550μL homogenization buffer (125mM Tris, 15mM MgCl2, 2.5mM EDTA pH 7.2, 1% Triton X-100, 1 tablet protease inhibitors [1697498, Roche, Indianapolis, IN] per 10mL buffer). Samples were agitated (20Hz) in chilled racks in TissueLyser II (QIAGEN) for three cycles of two minutes. Debris were pelleted by centrifugation, and supernatants were collected.

Multiplexed immunoassays

Levels of immune factors were measured in 40μL undiluted stool homogenates using the V-PLEX Neuroinflammation Panel 1 (human) Kit (Meso Scale Discovery, Rockville, MD) according to the manufacturer's protocol. This kit is divided into 5 panels of analytes grouped into the physiological categories "Angiogenesis," "Chemokine," "Cytokine," "Proinflammatory," and "Vascular Injury." Analytes measured were: vascular endothelial growth factor receptor 1 (Flt1), placental growth factor (PIGF), tyrosine kinase 2 (Tie-2), vascular endothelial growth factor A (VEGF), vascular endothelial growth factor D (VEGF-D), basic fibroblast growth factor (bFGF), eotaxin (CCL11), eotaxin-3 (CCL26), interferon gamma-induced protein 10 (IP-10, CXCL10), monocyte chemoattractant protein 1 (MCP-1, CCL2), monocyte chemoattractant protein 4 (MCP-4, CCL13), macrophage-derived chemokine (MDC, CCL22), macrophage inflammatory protein 1 alpha (MIP-1α, CCL3), macrophage inflammatory protein 1 beta (MIP-1β, CCL4), thymus and activation-regulated chemokine (TARC, CCL17), interleukin 12/interleukin 23 p40 (IL-12/IL-23 p40), interleukin 15 (IL-15), interleukin 16 (IL-16), interleukin 17 A (IL-17A), interleukin 1 alpha (IL-1α), interleukin 5 (IL-5), interleukin 7 (IL-7), lymphotoxin alpha (LTA), vascular endothelial growth factor C (VEGF-C), interferon gamma (IFNγ), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8, CXCL8), tumor necrosis factor (TNF), C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1).

Analyte levels were measured on the Meso Scale Discovery (MSD) QuickPlex instrument and evaluated on the MSD software platform. Values were normalized to total protein measured in each sample by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to manufacturer's protocol.

Statistical analysis

Chi square tests (two-tailed) were utilized to assess relationships between disease status (PD patients/controls) and discrete-valued (e.g., Yes/No) responses to various health-related questions. T-tests were used to compare levels of stool analytes between PD patients and controls, and Wilcoxon rank sum test was used to compare levels of stool analytes between

males and females (t-test deemed inappropriate due to data skew). General linear models (GLM) were used to investigate relationships between disease status and metadata factors. Multivariate analysis was performed using multiple regression GLM with multiple imputation (5) with the following potential confounders or effect modifiers: age, sex, geographic location, antibiotic use in the three months prior to stool collection, use of anti-inflammatory drugs which in these subjects included non-steroidal anti-inflammatory drugs or prednisone at least thrice a week (included automatically); body mass index (BMI), smoked at least 100 cigarettes in lifetime, current probiotic use including probiotic supplements or yogurt with live culture (included due to strong effects on stool analyte levels); birth by Caesarean section, digestive problems on the day of stool collection, diarrhea in the three months prior to stool collection, constipation in the three months prior to stool collection, coffee consumption, and alcohol consumption (included because incidence differed significantly between PD patients and controls). Unless otherwise specified, analyses were conducted using PROC GLM and PROC MIXED in SAS/STAT® software v9.3 for PC (SAS Institute Inc. 2011, SAS/STAT® 9.3 User's Guide. Cary, NC: SAS Institute Inc).

Results

Increased incidence of psychological and gastrointestinal symptoms in PD patients and decreased coffee and alcohol consumption

As expected, PD patients in this study reported increased incidence of anxiety (11 Controls-10.0%, 41 PD-26.1%, X^2=11.52, p=0.0007), depression (24 Controls-21.8%, 52 PD-33.1%, X^2=5.147, p=0.0233), and sleep problems (14 Controls-12.7%, 61 PD-38.9%, X^2=22.61, p=0.0001) (Table 1). Intestinal disease, which included inflammatory bowel disease, irritable bowel syndrome, Crohn's disease, and colitis, was also more common among PD patients (9 Controls-8.2%, 26 PD-16.6%, X^2=4.390, p=0.0361) (Table 1). There was no difference in subjects' reports of ulcers (Supp Table 3). Over 60% of PD patients who responded to the question reported experiencing digestive problems in the three months prior to stool collection, significantly more than controls (40 Controls-36.4%, 101 PD-64.3%, X^2=22.69, p<0.0001) (Table 1). This difference was driven by increased incidence of constipation in the past three months (4 Controls-3.6%, 65 PD-41.4%) as well as increased incidence of bloating (2 Controls-1.8%, 15 PD-9.6%) and excessive gas (2 Controls-1.8%, 21 PD-13.4%) on the day of stool collection, and it persisted despite decreased incidence of diarrhea over three months (27 Controls-24.5%, 21 PD-13.4%). PD patients were also more likely than controls to report taking medication for digestive problems (16 Controls-14.5%, 47 PD-29.9%, X^2=8.086, p=0.0045) (Table 1).

Among our subjects, there was a significant association between reported coffee consumption and disease status (X^2=11.61, p=0.0205), with controls reporting greater coffee intake (median 1–2 cups a day) than PD patients (median 2–6 cups a week) (Table 1). The same pattern was observed for alcohol consumption (X^2=19.38, p=0.0016) (Table 1). No differences were found between PD patients and controls with regard to smoking history, use of anti-inflammatory drugs, antibiotics, or probiotics. There were also no significant dietary

differences, although trends for reduced consumption of vegetables/fruits (X^2=5.365, p=0.0684) and nuts (X^2=7.353, p=0.0614) among PD patients were present (Supp Table 3).

Significantly higher levels of select stool analytes in PD patients

We first performed a direct comparison of immune- and angiogenesis-related analyte levels in the stool samples from PD patients and controls. Mean levels of 34 of 37 factors were higher in patients than controls, but three, Flt1, IL-1α, and CXCL8, met the threshold for significance ($p<0.05$) (Table 2).

To better account for the effects of subjects' environments on stool analyte levels, we performed a paired comparison of PD patients and their respective non-PD spouses. When all PD patients were compared with all household controls by paired t-test, there were no significant differences in levels of immune or angiogenesis factors. When patient-spouse pairs were separated by patient sex, however, a different pattern emerged. Male PD patients still exhibited no significant differences compared to their female spouses, but female PD patients had higher levels of 11 cytokines, chemokines, and angiogenesis factors compared to male spouses (Table 3).

Sex significantly impacts stool analyte levels

The household control comparison suggested that subject sex greatly impacted levels of immune and angiogenesis factors in stool. Indeed, when analyte concentrations between female and male subjects were compared, females exhibited significantly higher levels of 21 of 37 factors (Supp Table 4). The relationships between sex and disease status for each analyte were largely consistent, but three factors, PIGF, CXCL10 (IP-10), and CCL2 (MCP-1), were found at higher concentrations in female PD patients compared to female controls while no differences were found between male patients and controls (Supp Fig 1).

BMI, smoking history, and probiotic usage strongly influence stool analyte levels

Other metadata variables also influenced stool analyte levels. Nine chemokines and angiogenesis factors decreased significantly with increasing subject body mass index (BMI) (Supp Fig 2). Subjects who reported smoking at least 100 cigarettes in their lives (~5 packs) had significantly lower levels of eight different cytokines and vascular factors in stool (Supp Fig 3), regardless of disease status. One classically proinflammatory cytokine, IL-6, was significantly reduced with increasing coffee consumption (Supp Fig 4). Consumption of probiotics or probiotic yogurt was associated with significantly higher levels of three analytes, TARC (CCL17), IL-7, and MIP-1β, regardless of disease status (Supp Fig 5a), but an additional eight factors were significantly elevated only in PD patients taking probiotics (Supp Fig 5b). Most of the analytes affected by probiotic use were chemokines.

PD-associated stool inflammatory profile emerges when other factors are accounted for

Given the profound impact of numerous metadata factors on levels of stool analytes, we decided to more specifically evaluate PD-associated inflammatory signatures using a multivariate GLM approach adjusting for 13 metadata parameters. In this analysis, PD disease status was significantly ($p<0.05$) associated with elevated levels of IL-1α, IL-1β,

CXCL8, and CRP in stool. Elevations in Flt1 and PIGF were also trending (p=0.0541). (Table 4).

PD-associated GI inflammation does not emerge only in advanced disease

Alterations in immune activity occur with aging[36], and age is the primary risk factor for development of PD. Additionally, whether intestinal inflammation and dysfunction represent an independent facet of PD pathology or simply a response to increasing neurodegeneration remains unclear. To explore these potential relationships, we evaluated the effects of subject age and PD duration on the levels of immune and angiogenesis factors. A GLM regression of PD duration (age at time of stool collection minus age of PD onset) versus stool analyte levels revealed no significant association for any analyte (Supp Table 5). Mean levels of immune and angiogenesis factors remained consistent from recently diagnosed subjects to individuals who had lived with PD for more than 30 years. A GLM analysis evaluating the effects of subject age and disease status on stool analyte levels identified significant decreases in VEGF-C and IL-6 with increasing age regardless of disease status (Fig 1). Significant differences in the relationships between age and analyte levels for PD patients and controls were identified for MCP-1, TNF, IL-10, and IL-4. Levels of these factors trended toward a decrease with age in PD patients but remained relatively constant with age in controls (Fig 1). While not significant, this same pattern was observed for the majority of analytes measured.

Discussion

PD patients in this study reported the increased incidence of psychiatric and GI symptoms that is typical of PD. Measurements of stool immune factors indicated that gastrointestinal inflammation is present in these PD patients. This supports and expands upon previous reports of PD-associated dysbiosis, GI dysfunction, and colonic inflammation.

When levels of stool analytes were directly compared in PD patients and controls, Flt1, IL-1α, and CXCL8 were found to be significantly elevated. PD-associated increases in CXCL8 and IL-1α persisted after adjusting for 13 potential confounders or effect modifiers. CXCL8 is a highly proinflammatory molecule with neutrophil chemoattractant as well as angiogenic properties[37]. IL-1α is a potent initiator of GI inflammation which triggers recruitment of myeloid cells and their production of additional proinflammatory mediators such as IL-1β[38]. Production of IL-1α and IL-1β are induced in inflammation in part through activity of the transcription factor NF-κB, and, in an amplification loop, they also stimulate NF-κB activity[38]. In fact, all the inflammatory factors identified in the multivariate analysis as elevated in PD patients – CXCL8, IL-1α, IL-1β, and CRP – are targets for NF-κB transcriptional activity[39–41], and, like IL-1 cytokines, CRP also promotes NF-κB activity[42]. Our results suggest that this immune signaling pathway may be dysregulated in PD, leading to excessive inflammation.

In the multivariate analysis, elevations in angiogenesis-promoting Flt1 (VEGF receptor 1) as well as PIGF in stool from PD patients were trending (p=0.0541) but not significant. Inflammation and angiogenesis are closely linked[43] and frequently co-regulated, e.g. by NFκB[44]. Increased pro-angiogenic factors including VEGF and PIGF have been reported in

cerebrospinal fluid from PD patients, and it has been suggested that this promotes the blood-brain barrier dysfunction that has been documented in PD[45, 46]. Further investigation will be needed to determine whether consistent increases in angiogenic factors are present in the intestine in PD patients and how these might contribute to GI pathology.

The fact that disease-associated patterns in levels of immune factors did not change with PD duration suggests that intestinal inflammation is not exclusively present in advanced disease. PD-associated increases in stool analytes were also not explained by advanced subject age, as, if anything, levels of most analytes trended toward a decrease with age in PD patients. These findings support the hypothesis that intestinal inflammation is an early manifestation of PD that could contribute to the development of neuropathology rather than an effect arising in response to extensive gastrointestinal neurodegeneration.

Direct comparison of levels of stool immune and angiogenesis mediators in PD patients and controls was complicated by other variables. For instance, females were found to have significantly higher levels of most analytes measured compared to males. The minimal number of significant sex-disease status interactions, however, suggests that there is little sex difference in the nature of the impact that PD has on the gut immune environment. Higher levels of immune factors in stool from female subjects, though, could possibly contribute to differences in the severity of symptoms in PD[47, 48].

Subject sex also influenced the paired comparison of PD patients with their non-PD spouses, with no differences found between male patients and their female spouses but significantly elevated levels of 11 cytokines, chemokines, and angiogenesis factors in female patients compared to their male spouses. If there were no differences in levels of stool immune and angiogenesis factors between patients and controls, then females should have exhibited significantly higher levels of many analytes regardless of disease status. Instead, only female PD patients exhibited significant elevations compared to their partners, while the levels of stool cytokines, chemokines, and angiogenesis factors in males with PD resembled their female spouses. This supports a disease-associated increase in numerous immune and angiogenesis mediators that persists in a shared environment.

We also observed several interesting patterns in lifestyle factors that can alter stool immune and angiogenesis factor levels. While there were no significant differences reported in diet between PD patients and controls, the trending reductions in fruit and vegetable and nut consumption in PD patients may warrant further investigation, particularly as the immunomodulatory and neuroprotective potential of these foods in the context of neurodegenerative disease is beginning to emerge[49, 50]. The inverse correlations between BMI and certain stool analyte levels identified in this study may also merit further inquiry. While increased levels of immune and angiogenesis factors have been reported in serum and adipose tissue of overweight and obese subjects[51, 52], reductions in serum bFGF and Flt-1 with increasing BMI, such as we observed in stool, have been found[53, 54]. Furthermore, while increased fecal calprotectin in obese subjects suggests intestinal inflammation[55, 56], the relationship between BMI and levels of other immune and angiogenesis mediators in stool has not been explored, and our results suggest that it is complex.

Coffee and alcohol consumption and smoking are all reportedly reduced in PD patients[57–61], but whether these findings indicate protective effects of these practices, disease-associated suppression of psychological reward mechanisms[59], or simply reduced fluid intake due to dysphagia in advanced disease[62] remains undetermined. We observed reduced current coffee intake in our cohort of PD patients as well as reduced current alcohol consumption, in agreement with previous reports. While coffee and caffeine are known to have potent neuroprotective and anti-inflammatory properties[57], this study found only a minimal effect of alcohol and coffee consumption on levels of stool immune mediators, with coffee intake inversely associated with IL-6 levels. We found no differences between PD patients and controls in smoking history, but we did observe that having smoked at least 100 cigarettes reduced levels of multiple immune factors in stool. While cigarette smoke may have numerous deleterious effects systemically and in the gut[63–65], nicotine is known to have anti-inflammatory effects mediated by nicotinic acetylcholine receptor signaling and resulting in, among other effects, inhibition of NFκB[66]. Our results indicate that this immunomodulatory activity is prominent in the human gut. Interestingly, the smoking-associated differences we observed persisted even though over 92% of respondents in this study who reported having smoked were not current smokers and had been non-smokers for an average of 36.6 years. This suggests that the modulation of intestinal immune function mediated by smoking may be at least semi-permanent, perhaps aided by lasting alterations in the microbiome[59] and by epigenetic modification[67].

The higher levels of chemokines and other inflammatory factors associated with probiotic use, especially in PD patients, could have several explanations. Probiotics are thought to stimulate mucosal immune activity, and they may increase the production of chemokines that recruit primarily tolerogenic cell types. We also noted, however, that nearly 60% of controls and 80% of PD patients who reported using probiotics also reported experiencing digestive problems, so it is possible that the elevated levels of immune factors could result primarily from chronic GI problems rather than probiotics taken for their alleviation. A few studies have reported that the use of probiotics can ameliorate constipation in PD patients[68, 69]; trials measuring biochemical as well as clinical responses to probiotics may clarify whether these are truly a beneficial treatment for individuals with PD. Furthermore, the reciprocal interactions between specific intestinal bacteria, whether transient probiotics or resident commensals, and intestinal immune responses in PD remain to be elucidated and will likely provide greater insight into mechanisms of disease pathology than studies of either factor in isolation.

Our study also corroborates the few reports[32–34] of associations between PD and intestinal disease, with significantly more PD patients than controls in our cohort reporting a history of inflammatory bowel disease, irritable bowel syndrome, Crohn’s disease, and/or colitis. The mechanisms responsible for this epidemiological overlap have not been determined. There may be shared genetic predisposition for PD and intestinal disease; variations in the LRRK2 and NOD2 genes are associated with both PD[70, 71] and Crohn’s disease[72–74]. Another possibility is that the chronic inflammatory responses involved in GI diseases promote neuroinflammation and PD-associated neurodegeneration, a concept that is beginning to be tested in animal models[7,19]. Enteric inflammation and other changes in the GI environment

in PD could also contribute to disruption of intestinal immune tolerance and trigger clinical intestinal disease.

This study provides evidence that classic inflammatory processes are overly active in the intestine in PD patients and do not arise only in advanced disease. These could promote systemic and neuroinflammation and, ultimately, parkinsonian neurodegeneration. Because the immune mediators found to be elevated in PD patients would be produced in response to diverse insults, their specificity as biomarkers for PD is limited. Prospective studies would also be needed to determine when these indicators of GI inflammation appear in relation to motor symptoms. However, in combination with key pieces of patient information, it is possible that levels of select immune factors in stool could enable identification of individuals at risk for development of PD. Understanding the connections between intestinal inflammation and systemic and neuroinflammation may yield new insight into the mechanisms of PD pathogenesis and guide future investigations into immunomodulatory therapy that could potentially slow progression of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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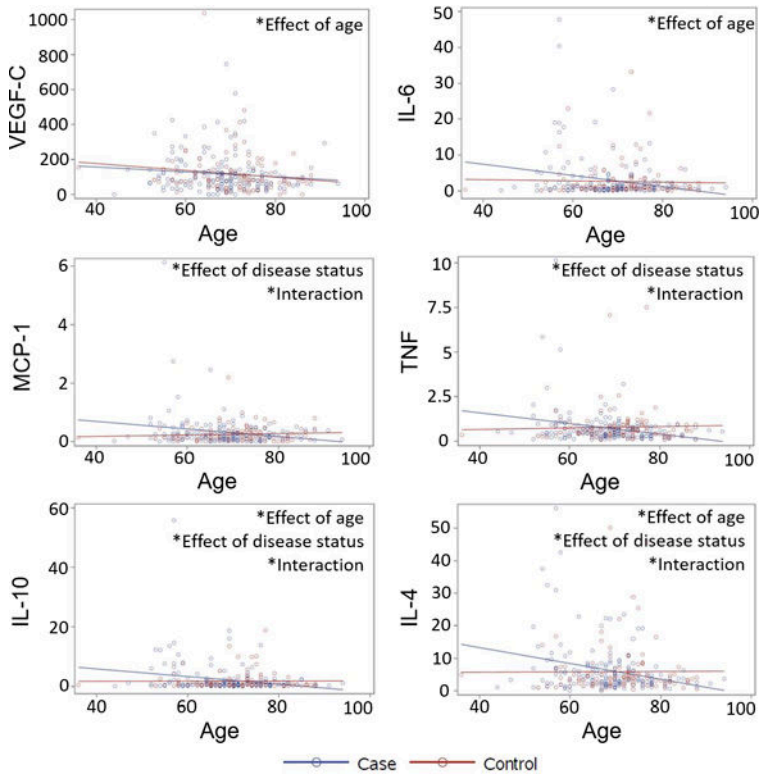

Figure 1. PD patients and controls differ in associations between subject age and levels of stool analytes

Levels (pg/mg) of analytes in stool homogenates from PD patients (blue) and controls (red) as a function of subject age. Significant ($p<0.05$) effects of age, disease status, and interactions between the variables by generalized linear model regression.

Mov Disord. Author manuscript; available in PMC 2019 May 01.

Table 1

Increased incidence of psychological and gastrointestinal symptoms in PD patients and decreased coffee and alcohol consumption

Question	Response	Subjects		X^2	p
		Controls	*PD Patients*		
Diagnosed or suspected anxiety	Yes	11	41	11.52	0.0007
		10.0%	26.1%		
	No	98	110		
		89.1%	70.1%		
Diagnosed or suspected depression	Yes	24	52	5.147	0.0233
		21.8%	33.1%		
	No	84	95		
		76.4%	60.5%		
Diagnosed or suspected sleep problems, insomnia	Yes	14	61	22.61	0.0001
		12.7%	38.9%		
	No	92	89		
		83.6%	56.7%		
Experienced digestive problems in the past 3 months	Yes	40	101	22.69	<0.0001
		36.4%	64.3%		
	No	58	40		
		52.7%	25.5%		
Currently on medication for digestive problems	Yes	16	47	8.086	0.0045
		14.5%	29.9%		
	No	89	106		
		80.9%	67.5%		
Diagnosed or suspected IBD, IBS, Crohn’s, or colitis	Yes	9	26	4.390	0.0361
		8.2%	16.6%		
	No	101	126		
		91.8%	80.3%		
How much caffeinated coffee do you drink	None	26	40	11.61	0.0205
		23.6%	25.5%		

Question	Response	Subjects		X^2	p
		Controls	*PD Patients*		
How much alcohol do you drink	<2 cups a week	10	27		
		9.1%	17.2%		
	2–6 cups a week	13	16		
		11.8%	10.2%		
	1–2 cups a day	36	55		
		32.7%	35.0%		
	3+ cups a day	25	14		
		22.7%	8.9%		
	None	31	59	19.38	0.0016
		28.2%	37.6%		
	< 2 drinks a week	47	48		
		42.7%	30.6%		
	2–6 drinks a week	8	31		
		7.3%	19.7%		
	1 drink a day	9	7		
		8.2%	4.5%		
	2 drinks a day	12	5		
		10.9%	3.2%		
	3+ drinks a day	3	2		
		2.7%	1.3%		

Table 2

Comparison of levels of immune and angiogenesis factors in stool from PD patients and controls

		PATIENTS			CONTROLS			
Panel	**Factor**	**N**	**Mean (pg/mg)**	**Std Dev**	**N**	**Mean (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	156	5.020	4.272	110	3.957	3.059	0.0184
	PlGF	156	1.439	1.132	110	1.352	0.6687	0.4328
	Tie2	156	194.2	152.1	110	181.3	103.7	0.4112
	VEGF-C	156	117.3	108.9	110	116.7	124.4	0.9662
	VEGF-D	156	19.49	14.50	110	19.39	11.32	0.9489
	bFGF	156	0.6937	0.5598	110	0.6165	0.4322	0.2040
Chemokine	**Eotaxin**	156	8.878	6.160	110	8.113	5.119	0.2697
	Eotaxin-3	156	24.05	19.06	110	22.35	20.77	0.4877
	IP-10	156	0.4657	0.4559	110	0.3964	0.2818	0.1265
	MCP-1	156	0.3225	0.5859	110	0.2562	0.2816	0.2195
	MCP-4	156	4.759	3.468	110	4.214	2.508	0.1366
	MDC	156	11.56	8.549	110	11.48	6.903	0.9306
	MIP-1α	156	4.879	4.204	110	4.363	3.370	0.2673
	MIP-1β	156	2.459	2.063	110	2.236	1.832	0.3616
	TARC	156	0.7362	0.6579	110	0.7790	0.5600	0.5781
Cytokine	**IL-12/23 p40**	156	2.634	1.779	110	2.481	1.522	0.4610
	IL-15	156	0.8354	0.6445	110	0.7519	0.6075	0.2857
	IL-16	156	16.34	17.50	110	15.28	14.17	0.5848
	IL-17A	156	2.955	8.555	110	2.104	1.452	0.2231
	IL-1α	156	98.99	326.8	110	41.65	40.39	0.0311
	IL-5	156	0.7553	0.8279	110	0.7062	0.6693	0.5926
	IL-7	156	0.7442	0.6159	110	0.6886	0.4264	0.3829
	LTA	155	0.4188	0.4499	110	0.4174	0.3734	0.9778
	VEGF	155	22.36	82.51	110	14.48	52.62	0.3423
Proinflammatory	**IFNγ**	155	11.93	27.88	110	9.68	14.49	0.3906

		PATIENTS			CONTROLS			
Panel	**Factor**	**N**	**Mean (pg/mg)**	**Std Dev**	**N**	**Mean (pg/mg)**	**Std Dev**	**p-value**
	IL-10	155	2.231	5.473	110	1.745	2.900	0.3487
	IL-13	155	3.463	2.437	110	3.255	1.880	0.4305
	IL-1β	155	3.451	12.22	110	1.685	2.486	0.0810
	IL-2	155	3.161	3.922	110	2.815	2.312	0.3664
	IL-4	155	0.6397	0.7944	110	0.5895	0.7576	0.6042
	IL-6	155	3.024	6.367	110	2.660	4.818	0.5951
	IL-8	155	41.5	149.1	110	14.0	53.5	0.0358
	TNF	155	0.7482	1.055	110	0.7787	0.9975	0.8121
Vascular Injury	**CRP**	155	1010	2909	110	547.4	1605	0.0978
	SAA	155	109.1	378.7	110	71.76	53.12	0.2257
	sICAM-1	155	19.87	44.79	110	13.22	41.83	0.2202
	sVCAM-1	155	22.26	22.09	110	22.61	21.50	0.8953

Mean protein levels (pg/mg total protein) with standard deviations (Std Dev) in stool homogenates

N = number of measurements for each subject group

Gray shading indicates significant ($p<0.05$) difference between patients and controls by t-test

Mov Disord. Author manuscript; available in PMC 2019 May 01.

Table 3

Comparison of levels of immune and angiogenesis factors in stool from PD patients and their spouses separated by sex

		PATIENT IS FEMALE				PATIENT IS MALE			
Panel	**Factor**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	10	1.776	3.077	0.1013	29	−0.3635	4.451	0.6635
	PlGF	10	0.6796	0.6393	0.0084	29	−0.2227	0.9099	0.1982
	Tie2	10	67.79	122.3	0.1135	29	2.117	168.1	0.9464
	VEGF-C	10	70.45	102.4	0.0575	29	−34.11	101.9	0.0824
	VEGF-D	10	8.259	9.029	0.0178	29	−3.290	16.87	0.3028
	bFGF	10	0.3046	0.3821	0.0327	29	−0.04650	0.7164	0.7291
Chemokine	**Eotaxin**	10	4.965	5.986	0.0277	29	−0.6213	6.545	0.6132
	Eotaxin-3	10	16.56	16.12	0.0100	29	−7.192	30.08	0.2084
	IP-10	10	0.2133	0.3305	0.0717	29	−0.01270	0.4037	0.8672
	MCP-1	10	0.1473	0.3382	0.2016	29	−0.03700	0.2909	0.4993
	MCP-4	10	1.897	3.314	0.1037	29	−0.7261	2.526	0.1328
	MDC	10	5.371	6.354	0.0255	29	−2.534	8.185	0.1066
	MIP-1α	10	3.501	5.582	0.0786	29	−0.2698	5.443	0.7914
	MIP-1β	10	1.538	2.092	0.0452	29	−0.2347	2.189	0.5683
	TARC	10	0.3986	0.3828	0.0093	29	−0.1299	0.7797	0.3773
Cytokine	**IL-12/23 p40**	10	0.6052	1.692	0.2874	29	0.04700	2.013	0.9007
	IL-15	10	0.3157	0.4257	0.0437	29	−0.01490	0.8733	0.9275
	IL-16	10	14.05	18.52	0.0399	29	−2.711	12.84	0.2652
	IL-17A	10	0.5709	1.475	0.2521	29	−0.02620	1.953	0.9429
	IL-1α	10	12.97	61.82	0.5236	29	165.9	569.2	0.1277
	IL-5	10	0.4791	0.5910	0.0305	29	−0.08360	0.7022	0.5268
	IL-7	10	0.1915	0.6243	0.3573	29	−0.07800	0.6125	0.4986
	LTA	10	0.1685	0.3000	0.1095	29	−0.1451	0.6120	0.2204
	VEGF	10	34.32	90.45	0.2608	29	10.35	61.63	0.3821
Proinflammatory	**IFNγ**	10	7.957	13.69	0.0993	29	5.571	49.55	0.5569

		PATIENT IS FEMALE				PATIENT IS MALE			
Panel	**Factor**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**
	IL-10	10	0.2590	1.876	0.6726	29	−0.08170	3.761	0.9093
	IL-13	10	0.5898	2.791	0.5208	29	−0.3801	1.872	0.2922
	IL-1β	10	1.973	6.809	0.3833	29	4.587	21.70	0.2731
	IL-2	10	0.7725	2.373	0.3301	29	−1.030	4.445	0.2307
	IL-4	10	0.02340	0.4506	0.8733	29	−0.1984	0.9819	0.2944
	IL-6	10	1.885	16.49	0.7262	29	−1.142	4.731	0.2124
	IL-8	10	4.943	22.29	0.5009	29	62.76	232.9	0.1654
	TNF	10	0.04880	0.5317	0.7782	29	−0.3561	1.385	0.1848
Vascular Injury	**CRP**	10	−671.7	2062	0.3298	29	933.4	4847	0.3173
	SAA	10	3.567	72.14	0.8792	29	−1.229	110.4	0.9534
	sICAM-1	10	1.930	4.425	0.2011	29	9.393	44.03	0.2689
	sVCAM-1	10	−1.153	14.72	0.8099	29	−5.996	36.20	0.3885

Mean difference (patient – control) between analyte levels (pg/mg total protein) with standard deviations

N = number of measurements for each subject group

Gray shading indicates significant ($p<0.05$) difference between patients and household controls by paired t-test

Mov Disord. Author manuscript; available in PMC 2019 May 01.

Mov Disord. Author manuscript; available in PMC 2019 May 01.

Table 4

Association between PD status and levels of stool immune and angiogenesis factors when accounting for potential confounders or effect modifiers

Panel	Factor	Estimate	95% Confidence Limits		p-value
Angiogenesis	**Flt1**	1.087	−0.01908	2.192	0.0541
	PlGF	1.087	−0.01908	2.192	0.0541
	Tie2	−0.4496	−39.62	38.72	0.9820
	VEGF-C	−9.056	−42.74	24.63	0.5968
	VEGF-D	−1.289	−5.128	2.551	0.5091
	bFGF	0.01910	−0.1271	0.1653	0.7971
Chemokine	**Eotaxin**	0.1777	−1.476	1.831	0.8325
	Eotaxin-3	0.9905	−4.683	6.663	0.7312
	IP-10	0.02551	−0.08694	0.1380	0.6554
	MCP-1	0.01348	−0.1242	0.1511	0.8471
	MCP-4	0.5197	−0.4079	1.447	0.2708
	MDC	−0.7171	−2.992	1.558	0.5353
	MIP-1α	−0.7171	−2.992	1.558	0.5353
	MIP-1β	0.08906	−0.4774	0.6555	0.7570
	TARC	−0.1315	−0.3080	0.04493	0.1433
Cytokine	**IL-12/23 p40**	0.02485	−0.4662	0.5159	0.9207
	IL-15	0.1547	−0.03270	0.3421	0.1052
	IL-16	1.367	−3.425	6.159	0.5747
	IL-17A	0.6550	−1.319	2.629	0.5139
	IL-1α	87.51	12.94	162.1	0.0216
	IL-5	−0.003209	−0.2306	0.2241	0.9778
	IL-7	−0.004470	−0.1665	0.1576	0.9567
	LTA	0.004860	−0.1218	0.1315	0.9398
	VEGF	19.25	−2.023	40.52	0.0759
Proinflammatory	**IFNγ**	3.607	−3.413	10.63	0.3124
	IL-10	1.296	−0.04960	2.641	0.0590
	IL-13	0.1148	−0.5554	0.7851	0.7360

Panel	Factor	Estimate	95% Confidence Limits		p-value
	IL-1β	3.015	0.2192	5.810	0.0347
	IL-2	0.6627	−0.3325	1.658	0.1908
	IL-4	0.06967	−0.1617	0.3010	0.5535
	IL-6	1.415	−0.2765	3.106	0.1007
	IL-8	40.28	4.368	76.19	0.0281
	TNF	−0.04665	−0.3570	0.2637	0.7674
Vascular Injury	**CRP**	1134	411.7	1857	0.0022
	SAA	1.357	−82.79	85.79	0.9747
	sICAM-1	7.169	−5.786	20.12	0.2767
	sVCAM-1	0.4134	−5.982	6.809	0.8988

Gray shading indicates significant ($p<0.05$) association with PD status parameter by multivariate GLM, multiple imputation = 5